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Effects of Female Wasp Accessory Secretions, Host Fat Body, and Host Hemolymph on Protein Synthesis and Egg Viability in *Microplitis croceipes* (Braconidae)

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Effects of female wasp reproductive gland secretions, host fat body and hemolymph, and mechanical constriction of the parasitoid egg on protein synthesis were studied in eggs of *Microplitis croceipes* (Braconidae) dissected from the wasp ovary. Protein synthesis was measured by ^{35}S-methionine incorporation in eggs held in tissue culture medium for 16 h after treatment. Synthesis was stimulated in oocytes obtained from three regions of the ovary (egg tube, reservoir, and calyx) by fat body and venom gland but not by calyx fluid. A combination of fat body, venom gland, and calyx fluid did not enhance the level of synthesis relative to that of fat body or venom gland alone. Host hemolymph inhibited protein synthesis when incubated directly with the dissected eggs but not when the eggs were collected from an artificial oviposition substrate (AOS) containing hemolymph. The inhibitory effect of the hemolymph is thought to be due to the occurrence of melanization. Mechanical constriction did not alter the rate of synthesis, confirming an earlier report that synthesis in newly deposited eggs is ongoing and is not dependent on mechanical activation during the act of oviposition. Mechanisms responsible for sustaining protein synthesis in eggs for 16 h in vitro after their exposure to host hemolymph in the AOSs or fat body and venom gland are not known. Only a small percentage (< 2%) of dissected ovarial reservoir oocytes that were mechanically constricted and exposed to the venom gland, calyx fluid, and host fat body hatched in vitro. In contrast, an earlier study demonstrated that 38% of eggs oviposited by female wasps into AOSs developed and hatched.

Key words: oviposition, insect reproduction, reproductive glands, parasitoid, parasite, venom gland

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INTRODUCTION

The eggs of hymenopteran parasitoids in comparison with free-living insects are unique in several aspects. The eggs are thin-walled, have little yolk when laid, and increase in size many times after being deposited into the host [1]. During oviposition, the egg is coated with a calyx fluid containing baculovirus-like particles and venom that are injected together with the egg [2]. Once the egg is deposited into the host, it is then bathed in the host's hemolymph, a hormonal and nutritional milieu that, in some cases, the endoparasitoid alters or modifies for benefit of its development [3]. The calyx and venom fluids have been studied primarily from the standpoint of their effects on the host, such as suppression of the immune response and also ecdysis of the host shortly after parasitoid oviposition [4–7].

The main focus of studies on the host's hemolymph has been on changes in hormonal titers, nutritional components, induction of new proteins [3,8–15]. An exception is an in vitro study on the direct growth-stimulating effects of host hemolymph on the endoparasitoids *Microplitis croceipes* and *Cotesia marginiventris* [16]. In the case of the endoparasitoid *Microplitis croceipes*, we have studied protein synthesis during growth and development of the maturing egg. Synthesis of proteins was evident as early as 1 h after the egg was deposited into the host [17]. We later found that synthesis did not depend on mechanical constriction of the eggs during oviposition but was a continuation of a similar level of protein synthesis occurring in the oocytes of the ovaries of *Microplitis* females [18].

Since the eggs of hymenopteran parasitoids are generally assumed to require passage through the ovipositor for development [19–21], mechanical activation may not be necessary to initiate protein synthesis; however, it may still be required to begin development. In this study we attempted to address three questions: 1) Will oocytes dissected from ovaries of female parasitoids initiate protein synthesis and develop similarly to naturally oviposited eggs? 2) Do female wasp accessory secretions and host hemolymph and/or fat body influence protein synthesis of eggs? 3) Do oocytes dissected from wasp ovaries require exposure to host hemolymph and/or host fat body to develop in vitro?

MATERIALS AND METHODS

Host and Parasitoid Colony Maintenance

The host species, *Heliothis zea* (Boddie), and the endoparasitoid *Microplitis croceipes* were reared as described earlier [17,22].

Basal Medium Preparation

Goodwin's IPL-52B medium [22] was obtained from Kansas City Biological Co. (Kansas City, MO) and prepared as described earlier [16]. For protein synthesis studies using radiolabeled methionine, the defined media were prepared without methionine and yeastolate.

Collection of Ovarial Eggs, Hemolymph, Calyx Fluid and Venom Gland; Preparation of Fat Body Conditioned Medium; and Mechanical Constriction of Eggs

Adult females 4–6 days old were removed from cages containing males and females. Females were submerged in 70% ethanol for 10 min, and their repro-

ductive tracts were dissected in Goodwin's culture medium and washed three times in fresh drops of media. The ovaries were dissected under a binocular dissecting microscope at 40× into three parts: egg tube, reservoir, and calyx. Eggs from the egg tube, reservoir, and calyx were each further dissected in separate drops of culture medium to prevent contamination of one from the other. Developing eggs of similar size were gently teased from the egg reservoir and calyx but were left intact in the egg tube. To standardize counting the eggs in the egg tube, only egg chambers that possessed a distinct opaque area were counted. The eggs and egg tube were rinsed by transferring them through three drops of media, and then they were transferred to 200 μl of media in a plastic petri dish (9 × 50 mm). A 20 μl microcapillary tube drawn out to a fine tip was used to transfer the eggs from one drop to another. The number of replicates ranged from 3 to 7, and each replication contained 40–60 oocytes. Data were analyzed with one-way analysis of variance and Duncan's multiple range test [23].

Hemolymph was collected from fifth instar *H. zea* larvae (average weight 340 mg), heat-treated at 60°C for 5 min or treated with PTU,* 0.05% final concentration to inhibit melanization, and subsequently diluted in half using IPL-52B defined media without methionine. This solution was then filter sterilized (Millex-GVO 0.22 μm syringe filter; Millipore, Bedford, MA), and one 200 μl drop was dispensed into each sterile culture dish. Dishes with heat-treated hemolymph were allowed to incubate for a period of 16 h; those with PTU had no period of incubation prior to the addition of 1 μl (4 μCi) ^{35}S-methionine (1,032 Ci/mmol) in aqueous solution (Amersham, Arlington Heights, IL).

Calyx fluid was collected from separate ovaries using a finely drawn 20 μl microcapillary tube attached to a 10 cc syringe by plastic tubing. Calyx fluid was incubated with eggs dissected from ovaries. Venom glands were dissected in Goodwin's culture medium from the reproductive organs of female wasps, rinsed three times with medium, and incubated with eggs dissected from ovaries.

For tests on effects of cocultured host fat body on protein synthesis, fourth instar host larvae (average weight 188 mg) were sterilized, and fat bodies were dissected as described earlier [16]. To precondition the culture medium, 15–20 mg of fat body was added to a 200 μl drop of medium and incubated at 28°C for 24 h. Methionine-free and yeastolate-free medium was also preconditioned with fat body for use in studies on effects of fat body on incorporation of ^{35}S-L-methionine into protein in developing eggs.

To determine whether reservoir eggs would continue developing in vitro without having to pass through the ovipositor, eggs were dissected from the ovarial reservoir and incubated in 100 μl of fat body preconditioned media. To determine effects of the venom gland and the calyx fluid on development, one venom gland and fluid from one calyx were placed in a media drop with the eggs. Eggs were observed daily over a period of 10 days, and the numbers attaining germ band stage, larval appendages, and hatch were recorded.

To determine if protein synthesis could be activated by mechanically constricting the eggs, both reservoir and calyx eggs were forced through a 25 μl

*Abbreviations used: AOS = Artificial oviposition substrate; JH = juvenile hormone; PTU = phenylthiourea; TCA = trichloroacetic acid.

capillary tube with the tip slightly narrowed. This resulted in a gentle constriction of the eggs during passage.

Preparation of AOS for Egg Collection

AOSs were prepared from agarose in the form of hardened gel drops treated with host hemolymph and presented to female wasps as described earlier [24,25]. To collect viable eggs from the AOSs, they were prepared from a gel solution containing Goodwin's isotonic IPL-52B tissue culture media.

Incorporation of ^{35}S-Methionine

Procedures described earlier for incorporation of ^{35}S-methionine were followed with slight modifications [18]. Dissected oocytes of *M. croceipes* were placed into 100 μl Goodwin's IPL-52B medium (minus yeastolate and methionine). Label was added in a 1 μl aqueous solution that contained 4 μCi ^{35}S-methionine (1,420 Ci/mmol; Amersham) for a period of 2 h at 28°C. After incubation with the label, oocytes were removed from the medium in a 50 μl aliquot and placed into 500 μl of Goodwin's IPL-52B medium (minus yeastolate) and incubated for 1 h at 27°C. Eggs were transferred to a microfuge tube containing bovine serum albumin (140 μg) as a coprecipitate in 50 μl of media and 200 μl 70% trichloroacetic acid (20% final volume). The tube was then sonicated for 2 min and centrifuged. This step was repeated twice, after which the TCA pellet was resuspended and sonicated in 1 ml of InStaGel (Packard). Radioactivity was measured with a Tri-Carb (4000)® liquid scintillation counter (95% counting efficiency).

RESULTS AND DISCUSSION

Influence of Hemolymph, Fat Body, Accessory Glands, and Mechanical Constriction on Protein Synthesis in Ovarial Dissected Eggs

Fat body and calyx fluid. Fat body is known to play an important metabolic role in the insect. The tissue is the principal synthetic source for storage proteins, lipids, carbohydrates, and transport proteins such as lipid and JH binding proteins [26]. In the endoparasitoid *Microplitis croceipes*, fat body from unparasitized host larvae of *Heliothis zea* was shown to promote in vitro hatching of pregerm band eggs of *M. croceipes* [16]. Therefore the influence of fat body on protein synthesis in eggs dissected from egg tube, calyx, and reservoir of the ovary was examined. In addition, the effect of calyx fluid plus fat body on protein synthesis was studied. Although interest in ovarial calyx fluid has been mainly because of its role in suppression of the host's immune system, it seemed reasonable to consider the influence of the secretion on protein synthesis since the fluid is injected along with the parasitoid egg during oviposition. Ovarian calyx fluid of *M. croceipes* contains virus particles similar to those seen in *Apanteles melanocelus*, a parasitoid of *Manduca sexta* [27]. The injected virus calyx particles were found in host fat body and muscle tissue within 2 h after oviposition and were thought to be involved in abrogation of the habitual host's defensive hemocytic reaction against the egg. Figure 1 shows the influence of fat body and calyx fluid preconditioned tissue media on protein synthesis in eggs held in the tissue media for 16 h. Media

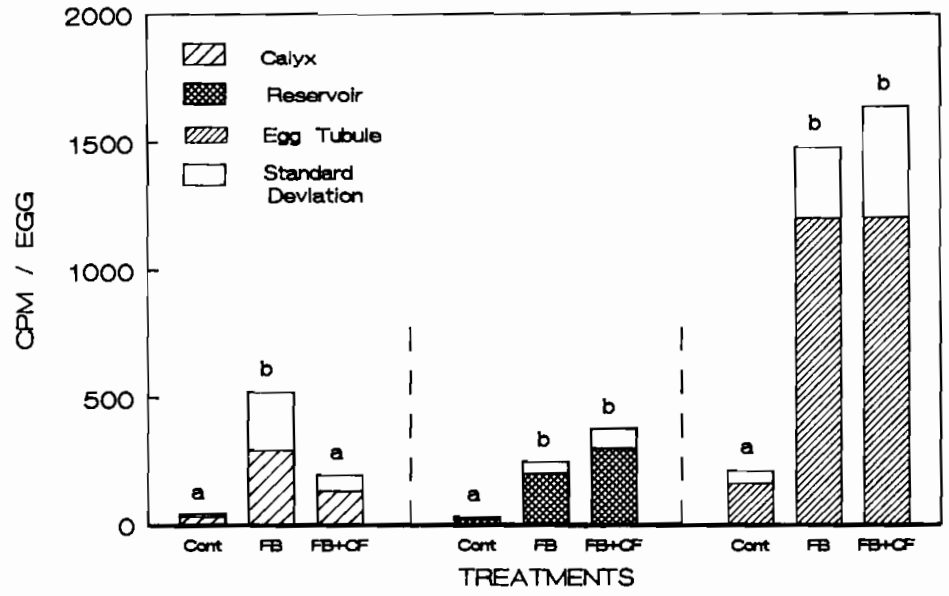

Fig. 1. Effect of wasp calyx fluid plus host fat body on protein synthesis in eggs held in the medium for 16 h after dissection from the egg tube, calyx, and reservoir of the ovary. **Cont,** control eggs; **FB,** fat body; **CF,** calyx fluid. Means followed by different letters above each bar within each group of oocytes are significantly different at $P = 0.05$.

preconditioned with fat body alone maintained a level of synthesis significantly higher than the control eggs. The fat body likely released into the media nutrients and/or growth-stimulating factors that were required by the egg for maintenance of protein synthesis. Adding calyx fluid to fat body, however, did not significantly increase protein synthesis in any of the three egg stages.

Venom. In endoparasitic insects, fluid from the venom gland is also injected along with the egg into the host [3]. To determine whether the venom gland fluid had any effect on protein synthesis, we incubated eggs from the calyx, reservoir, and egg tube of the ovary with the venom gland for 16 h before measuring the level of ongoing synthesis in the egg (Fig. 2). The venom gland was comparable to host fat body in stimulating protein synthesis in the three egg stages (Fig. 2). Incubating fat body plus venom gland together with reservoir eggs significantly increased synthesis over that of either fat body or venom gland alone in the reservoir and egg tube eggs. Perhaps of greatest interest is the stimulation of protein synthesis by venom gland alone in the reservoir and egg tube oocytes. The venom gland of Braconid wasps was reported to contain polypeptides and pharmacologically active substances [28], and in *M. croceipes* (personal observation) the gland contains peptidases that are capable of digesting gelatin and host hemolymph proteins. Possibly a protease in the venom acted on the egg membranes and catalyzed protein synthesis in the eggs. Proteases, generally known for their biological function as hydrolases, also have been reported to catalyze the reverse reaction, the formation of peptides [29]. Another possibility is that a low-molecular-weight substance released by the gland and taken up by the egg stimulated protein synthesis. This seems plausible since the chorion of most endoparasitoids eggs is thin-walled and is permeable to ions and low-molecular-weight molecules [1,17].

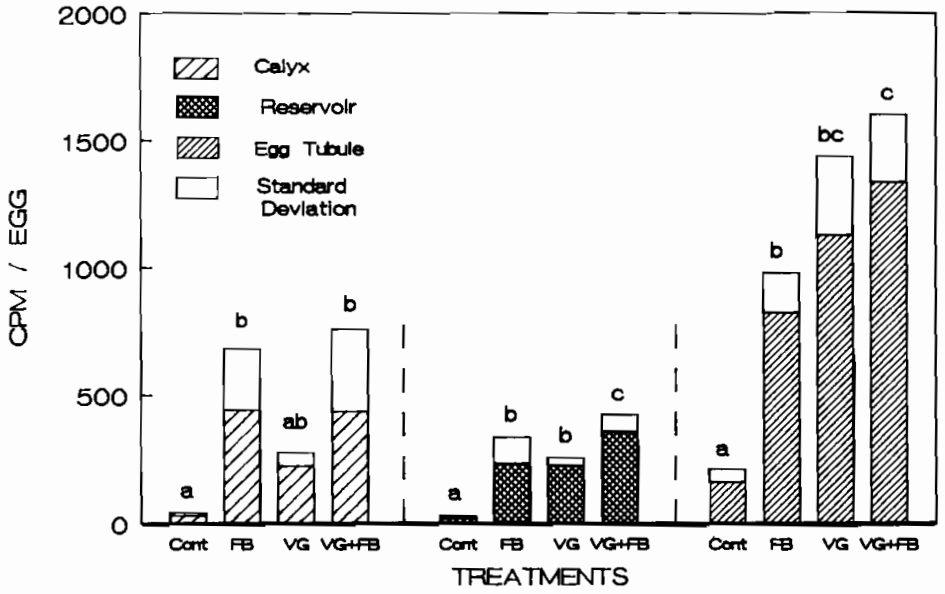

Fig. 2. Effect of venom gland plus fat body on protein synthesis in eggs held in medium for 16 h after dissection from the egg tube, calyx, and reservoir of the ovary. VG, venom gland. Means followed by different letters above each bar within each group of oocytes are significantly different at $P = 0.05$.

Fat body, calyx, and venom combination. In studies on suppression of the host's immune system, venom was reported to enhance the viability of the injected virus in *Cotesia melanocelus* by uncoating the nucleocapsids at the nuclear pores of the host cells and altering virus uptake by the cells [30]. Also, venom of *A. glomeratus*, in conjunction with calyx fluid was important in suppressing encapsulation of the parasitoid eggs in *Pieris rapae crucivora*. Venom was thought to interact with the egg surface and in some way prevent host hemocytes from reacting against the parasitoid egg [31]. We thus addressed the question of whether the combination of venom gland incubated with fat body plus calyx fluid would influence the level of protein synthesis. Figure 3 shows that the combination of fat body, venom gland, and calyx fluid did not affect the level of protein synthesis relative to that of fat body alone.

Mechanical constriction. Our earlier studies with *M. croceipes* indicated that initiation of protein synthesis was not dependent on mechanical constriction of the eggs during oviposition, because the level of synthesis in eggs dissected from the ovaries immediately before oviposition was similar to that of eggs oviposited into the host [17]. However, in this study we wanted to know if constricting eggs dissected from the ovary would help maintain synthesis over the 16-h incubation period. Mechanically constricting oocytes from the reservoir and calyx of the ovary resulted in highly variable data, despite our attempts to keep variability at a minimum by carefully repeating the method of constricting the oocytes. Nevertheless, the means and standard deviations of cpm/egg were 429 ± 374 and 491 ± 357, respectively, for control and constricted calyx oocytes; and 259 ± 159 and 256 ± 175, respectively, for control and constricted reservoir oocytes. Therefore, constricting the eggs did not appear to influence the level of protein synthesis while they were held in vitro for 16 h.

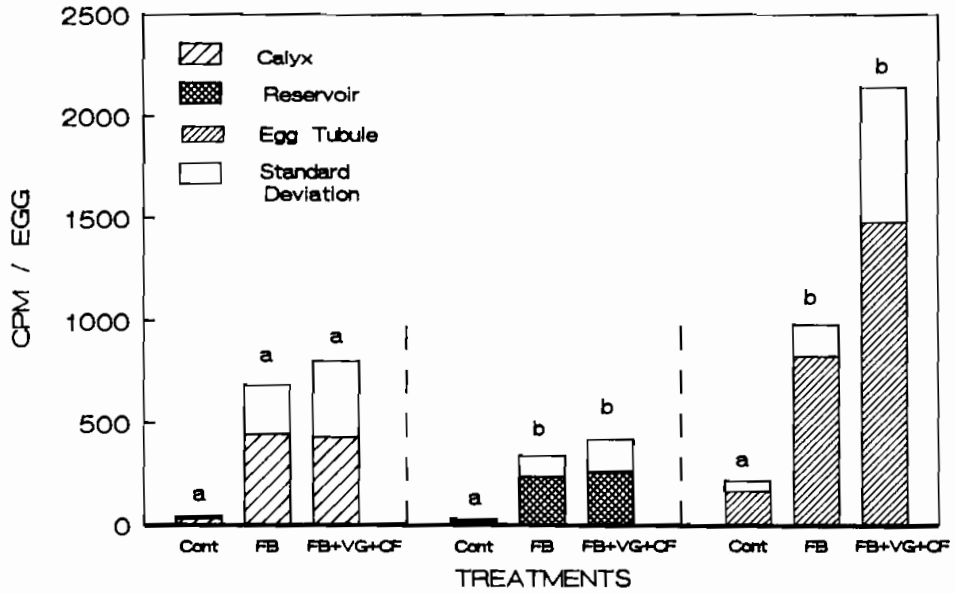

Fig. 3. Effects of combination of calyx fluid, venom gland, and fat body on protein synthesis in eggs held in the medium for 16 h after dissection from the egg tube, calyx, and reservoir of the ovary. Means followed by different letters above each bar within each group of oocytes are significantly different at $P = 0.05$. Abbreviations same as in Fig. 1.

Eggs Dissected From AOS

Use of an AOS provided eggs that were naturally exposed to host hemolymph and to the calyx and venom gland fluids and to mechanical constriction during the act of oviposition. This allowed a comparison of naturally oviposited eggs with eggs dissected from the ovaries and exposed to the various female accessory fluids as discussed above. Protein synthesis in eggs dissected from AOSs containing host hemolymph and held in medium for 16 h in vitro had a specific activity of 961 ± 355 (SD) cpm/egg. This rate of synthesis for oviposited eggs was comparable to that for dissected calyx eggs held with fat body in vitro for 16 h (see Fig. 1). Eggs collected using the AOSs were exposed to host hemolymph for up to 3 h, the length of time the females were allowed to oviposit into the substrates. Apparently the hemolymph did not melanize in the AOSs, and this period of exposure to the hemolymph and/or the act of oviposition provided the mechanical and/or chemical stimuli needed for maintaining protein synthesis. When we tested hemolymph in vitro with dissected calyx, reservoir, and egg tube oocytes, however, protein synthesis was inhibited rather than maintained for 16 h as in the AOS-collected eggs. Greany [16] reported that newly laid eggs of *M. croceipes* did not complete development in vitro when placed in any concentration of hemolymph from nonparasitized *H. zea*. The author concluded (personal communication) that melanization of hemolymph was responsible for failure of the eggs to develop. This is likely the case, since Greany et al. [32] more recently have shown that a phenolase-free hemolymph fraction containing protein stimulated egg development in vitro.

Influence of Accessory Gland Secretions on In Vitro Growth and Development of Dissected Ovarial Reservoir Eggs

It is generally assumed that eggs must be constricted during oviposition for development of the egg to proceed [19–21]. In this study, however, the venom gland stimulated protein synthesis in vitro in eggs that were not constricted. The next question that arose was, Will the venom gland secretions also influence growth and development of *Microplitis* eggs that are not constricted? To address this point, eggs from the ovarial reservoir were incubated with fat body and accessory/gland secretions in tissue culture medium. It is interesting that a small number of eggs exhibited some development, and a few actually hatched in the medium; however, the number was so low that no comparisons in regard to hatch could be made between the various treatments (Table 1). Comparing these data with those from an earlier report [25] that viable eggs could be collected from females of *M. croceipes* using an AOS and host hemolymph, it appears that the egg may need to be mechanically activated and/or may require exposure to some component in the host's hemolymph to be developmentally activated. Eggs of *M. croceipes* dissected from host larvae were found to be dependent on a host hemolymph protein for initiation of embryogenesis in vitro [32]. Fat body tissue could be substituted for hemolymph in inducing egg development, suggesting that the fat body may release the growth-stimulating factor into the hemolymph [16]. Eggs of *Campoletis sonorensis* removed from the lateral oviducts of females did not initiate development upon injection into the host unless the eggs were mechanically stressed or exposed to hypertonic insect salines and cold shock [21].

Possible Explanation for Effects of Wasp Venom Gland and Host Fat Body and Hemolymph on Protein Synthesis In Vitro

Our results indicate that fat body and venom gland stimulated protein synthesis in eggs that were dissected from ovaries and held in vitro. Hemolymph apparently inhibited protein synthesis because of toxic products formed during melanization during both 2- and 16-h incubation periods. However, hemolymph did stimulate synthesis in eggs collected using the AOSs. AOSs stabilized the hemolymph during the 3-h period used to collect eggs, and this brief exposure to the hemolymph in the AOSs was adequate to maintain protein synthesis at a rate comparable to that in eggs maintained in eggs held in fat body conditioned medium.

TABLE 1. Development of *M. Crocepies* Reservoir Eggs Dissected From the Lateral Oviduct and the Influence of Ovarial Calyx Fluid and Venom Gland on Development in Fat Body Preconditioned Tissue Culture Medium

Treatment	No. of replications	No. of eggs	Germ band (%)	Embryos (%)	Hatch (%)
Reservoir eggs	3	53	6.3	4.8	1.5
Reservoir eggs + calyx fluid	5	108	2.6	0	2.0
Reservoir eggs + venom gland	3	70	1.3	1.3	0
Reservoir eggs + calyx fluid + venom gland	5	113	1.6	1.6	1.6

There are probably a number of reasons why each of the three tissues stimulated protein synthesis. One possibility is that an initiation factor for protein synthesis was released into the tissue culture medium. Soluble initiation factors have been extensively studied in various mammalian systems, and the tissues may have contributed an initiation-like factor that participates in formation of initiation complexes or mRNA binding proteins [33–35]. It is also possible that dissociation of an inhibitor was involved in activation of translation of maternal mRNA, as has been reported in oocytes of *Dysdercus intermedius* and *Smittia* species [36,37]. In the case of *M. croceipes*, digestion of the inhibitor might occur because proteases present in the venom gland fluid (personal observation) are injected into the host along with the egg during oviposition. This view is plausible because protein synthesis in *M. croceipes* eggs held in the egg reservoir and calyx of the ovary and during early embryogenesis was attributed to translation of stable maternal RNA originating in nurse cells during oogenesis [18].

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